EFFECTS OF ORALLY ADMINISTERED UNDENATURED TYPE II CHICKEN COLLAGEN AGAINST ARTHRITIC INFLAMMATORY PATHOLOGIES: A MECHANISTIC EXPLORATION

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Summary: *Arthritis afflicts ~43 million Americans or approximately 16.6% of the U.S. population. The two most common and best known types of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). A significant amount of scientific research has been done in attempts to explain what initiates forms of arthritis, how it is promoted and perpetuated, and how to effectively intervene in the disease process and promote cartilage remodeling. Current pharmacological strategies mainly address immune suppression and anti-inflammatory mechanisms, and have had limited success. Recent research provides evidence that alterations in the three dimensional configuration of glycoproteins are responsible for recognition/response signaling that catalyzes T-cell attack. Oral administration of auto-antigens has been shown to suppress a variety of experimentally induced autoimmune pathologies, including antigen-induced RA. The interaction between Gut Associated Lymphoid Tissue (GALT) in the duodenum and epitopes of orally administered undenatured type II collagen facilitates oral tolerance to the antigen and stems systemic T-cell attack on joint cartilage. Previous studies have shown that small doses of orally administered undenatured type II chicken collagen effectively deactivate killer T-cell attack. A novel glycosylated undenatured type II collagen material (UC-II) was developed to preserve biological activity. The presence of active epitopes in the UC-II collagen is confirmed by an ELISA test and distinguishes this form from hydrolyzed or denatured collagen. Oral intake of small amounts of glycosylated UC-II presents active epitopes, with the correct three-dimensional structures, to the Peyer's Patches, which influences the signaling required for the development of immune tolerance. UC-II has demonstrated the ability to induce tolerance, effectively reducing joint pain and swelling in RA subjects. A pilot study was conducted for 42 days to evaluate the efficacy of UC-II (10 mg/day) in five female subjects (58-78 yr) suffering from significant joint pain. Significant pain reduction including morning stiffness, stiffness following periods of rest, pain that worsens with use of the affected joint and loss of joint range of motion and function were observed. Thus, UC-II may serve as a novel therapeutic tool in joint inflammatory conditions and symptoms of OA and RA.*

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Introduction

Arthritis represents a group of debilitating diseases of the joints, bones, tendons, muscles and eventually organs. It afflicts approximately 43 million Americans, imposing a cost in excess of $65 billion annually[1]. The two most common types are osteoarthritis (OA) and rheumatoid arthritis (RA), traditionally defined as age related "wear-and-tear" arthritis and "auto-immune" arthritis, respectively[1,2]. However, inflammatory response has been identified to be a common mediator in both types of arthritis[1,2].

The understanding of RA pathogenesis has changed over the years. RA is characterized by attack of killer T-cells on type II joint collagen, which results in damage to cartilage, joint swelling, pain and inflammation[3-6]. The body's attempts to remodel joint cartilage are outpaced by immune mediated attack on and degradation of joint cartilage[3-6]. Collectively, these events have been characterized as an out-of-control autoimmune response[3]. Extensive research has explored the multifaceted dynamics of recognition, response, and compensatory homeostatic mechanisms in an effort to understand, manage and maintain immune competence. Research in transgenic mice points to the possibility that B-lymphocytes and immunoglobulins outside the joint indirectly provoke RA pathogenesis via a self-reactive T-cell receptor in the joint[7]. However, our understanding of autoimmunity still presents unresolved challenges that may require a paradigm shift in research for the development of effective and safe therapies.

Immune Behavior and The Aging Paradox. It has been proposed that mechanisms involved in host defense, protection and maintenance of self-integrity are counteracting forces in which tolerance mechanisms efficiently suppress immune attack on self to a required threshold. An evolutionary perspective alleges that a tendency toward autoimmune malfunction should theoretically be higher during years when young immune systems are aggressively protecting the reproductive potential of the host. Mis-recognition of self would be a predictable deficiency of the system[8]. Autoimmune disorders are less prevalent in the young, increasing with advancing age and decline of reproductive potential. In fact, there is a clear relationship between advancing age and increased incidence of arthritis. To explain this, one rationale theorizes that RA pathology therefore, must result from a deteriorating function of the immune system, which provides ideal conditions for a breakdown in self-tolerance[8]. Decreased recognition and up regulated self-attack is a logical conclusion consistent with age-related decline in immune efficiency[9]. However, explanations regarding "auto-reactivity" of the immune system in RA pathology favor an emphasis on functional flaws in surveillance, recognition and response (and their symptomatic manifestations) rather than the possibility that structural flaws in immune system complexes, and possibly the target tissues, may be etiological catalysts. Recent strategies for therapeutic management of RA therefore, focus on methods of inhibiting symptom manifestation to reduce the severity of the end-stage of this disease[10].

Etiology and Pathogenesis of RA. Etiological and therapeutic research faces the challenge of explaining how the arthritic processes originate and progress[2]. Most of the past and current work on rheumatoid pathologies examine strategies to intervene or halt "out of control" immunologic and/or inflammatory events associated with autoimmune disease[10]. The traditional paradigm proposes that RA is an immunological disorder for an, as-yet-unidentified, arthritogenic antigen. Various immunological factors are involved, such as CD4-inducer lymphocytes, CD4 cells, macrophages, neutrophils and tumor necrosis factor (TNFα)[9,10]. This conventional view has produced pharmacological therapies that favor manipulation of COX-2 events and immune suppression, with less than ideal results. Almost all of the biomolecules responsible for innate and adaptive immune response are glycoproteins[11]. However, little attention is directed at the possibility that impaired glycosylation affects the three dimensional configuration of glycoproteins, including IgG and type II collagen. These may alter recognition and response signaling during immune surveillance, inciting attack on the body's own joint collagen[11-19].

This view suggests that perceiving RA as a hyperreactive "immune abnormality" may be a misnomer, as the immune system is behaving appropriately against host tissues ultimately identified as foreign pathogenic antigens[10]. Altered glycosylation could produce a number of identification errors responsible for up regulating self-attack. Among the possibilities are: misidentification of Type II joint collagen as antigenic by aberrant IgG; possible binding of hypogalactosylated IgG with certain Rheumatoid Factors (RF) leading to significant levels of immune complexes characteristic of RA; and/or appropriate glycomic identification markers may be missing from the joint collagen itself. This perspective provides insight into how the immune system incurs a loss of self-tolerance and explores the possibility of flaws in glycosylation/galactosylation. This phenomenon is the root of impaired immunological recognition and response activities for the hyper-auto-reactive immune self-destruction of joint collagen in the pathogenesis of RA[19,20]. Hence, alterations in glycosylation/galactosylation are hallmark characteristics of RA. This also provides a possible explanation as to why orally ingested native type II collagen produces tolerance, down regulating autoimmune aggression[3,4].

Impaired galactosylation affects glycoprotein synthesis, altering the requisite three-dimensional conformations of glycoproteins such as type II collagen and IgG, producing the loss of self-recognition. Lang and Yeaman (2001) demonstrated that removal of carbohydrate moieties from antigens resulted in a loss of antibody binding[20]. In RA patients, decreased levels of β1-4 galactosyltransferase (GalTase) activity in peripheral blood B- and T- Lymphocytes correlates with the decreased galactosylation of serum IgG[13].

Immunoglobulins are by definition glycoprotein molecules produced by plasma cells in response to an immunogen, which function as antibodies[11]. In RA, immune complexes are present that consist exclusively of immunoglobulin, indicating a role as both an antibody and antigen. Both cartilage and immune system complexes are, for the most part, made of glycoprotein structures in which glycoprotein synthesis requires the necessary substrate and competent glycosylation[16]. Impaired glycosylation/galactosylation intersects at a number of junctures contributing to the initiation, promotion and progression stages of RA[11-19].

Comparisons of the N-glycosylated pattern of serum IgG isolated from normal individuals with that of RA patients demonstrates that differences observed in RA patients are due to changes in the relative extent of glycosylation compared with normal individuals. In RA, an increased number of oligosaccharide structures lack the terminal galactose residue[19]. This suggests that RA may be a glycosylation disease, reflecting changes in the intracellular processing, or post secretory degradation of N-linked oligosaccharides[12,19]. Other research has reported a decrease in galactose residues in the oligosaccharide chains of the serum IgG of RA patients, which was presumed to affect the three-dimensional structure of the CH2 domain. Galactose depleted IgG reduced Clq binding and Fc receptor binding, which imply an important biological function to the glyconutrient moiety of IgG[16]. Rademacher, et al., (1994) demonstrated clear evidence that galactose deficient IgG glycoforms are directly associated with pathogenicity in collagen induced rheumatoid arthritis in mice[17]. Nonpathogenic autoantibodies were made pathogenic by altering their glycosylation state[17].

Oral Tolerization. Immunization with undenatured type II collagen (antigen) has been shown to induce arthritis[21]. However, orally ingested undenatured native antigens interact with gut associated lymph tissue (GALT), resulting in an entirely opposite effect. Oral tolerization, using small doses of UC-II, has demonstrated its effectiveness in turning off T-cell attack on type II joint collagen, inducing immunological hyporesponsiveness, and reducing pain and inflammation[3-6]. In contrast, while denatured collagen may provide a nutritional source of substrate for joint cartilage synthesis, research demonstrates that it does not induce immunological hyporesponsiveness and has not demonstrated an effect on reducing pain and inflammation[6]. Although the same amino acids are present in both forms, the tertiary and quarternary structures in the denatured form may be completely destroyed and the galactose moiety is degraded (Figure 1), not allowing epitope recognition in the Peyer's Patch[3,10,22]. Furthermore, the hydrolyzed or denatured form may be pharmacologically ineffective because of the loss of conformation. Interestingly, the effects of oral tolerance do not appear to be confined to RA pathologies alone, but confer appreciable benefits in some cases of OA as well. A pilot study provides preliminary evidence that 10 mg/day of a commercial ELISA verified undenatured glycosylated type II collagen (UC-II™ InterHealth Nutraceuticals Incorporated, Benicia CA) administered orally reduced sensory pain by 26% in 4 out of 5 females, age 58-78 years, for 42 days. Two of the women were previously diagnosed with OA and the remaining 3 exhibited similar symptoms but had no clinical diagnosis. There were no adverse side effects associated with the intake of UC-II (Table 1).

Role of Peyer's Patches in Oral Tolerance. Peyer's Patches are relatively large aggregates of lymph tissue located in the GALT of the small intestine[10,22]. The overlying 'dome' epithelium contains large numbers of intraepithelial lymphocytes. Some of the epithelial cells have complex microfolds in their surfaces, known as M-cells. M-cells are important in the transfer of antigen from the gut lumen to the Peyer's Patch[10]. Peyer's Patches then facilitate the generation of an immune response within the mucosa. An antigen in the Peyer's Patch stimulates B-cell precursors and memory cells[10]. Cells pass to the mesenteric lymph nodes where the immune response, if needed, is amplified. Activated lymphocytes pass into the blood stream via the thoracic duct. Oral tolerance

only occurs after the correct three-dimensional conformation of UC-II antigen is identified as nonpathogenic[10,22].

Materials and Methods

Chemicals. Pepsin (Catalog # I.U.B. 3.4.23.1) was purchased from Worthington Biochemical Corporation (Freehold, NJ). Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Undenatured type II collagen (UC-II). UC-II was obtained from InterHealth Nutraceuticals (Benicia, CA). The presence of glycosylated "active" epitopes in the UC-II collagen matrix was confirmed by a validated ELISA test. Furthermore, electron microscopic analysis of UC-II was conducted to demonstrate the conformational integrity of the triple helical structure.

For electron microscopic analysis, a small amount of UC-II powder was fixed with Karnovsky fixative for 2 hr, rinsed with cacodylate buffer for 20 min, placed in 1% osmium tetroxide for 2 hr, rinsed with distilled water for 1 min, and placed overnight in 0.5% uranyl acetate. The sample was then dried using ethanol and placed into propylene oxide for 30 min, and finally placed in 50:50 propylene oxide:SPURR (embedding material) for 2 hr and then into 100% SPURR overnight. It was then placed into a 70ºF oven overnight. A section was taken using ultra microtome, stained with uranyl acetate for 4 min, rinsed with distilled water, stained with lead citrate for 2 min, and rinsed again with distilled water and dried. The transmission electron microscope procedure was conducted in an EM JEOL 100CX (Peabody, MA). Electron micrograph of undenatured type II collagen vs denatured type II collagen is shown in Figure 1. Undenatured type II collagen (on left) shows intact tertiary and quarternary glycoprotein integrity allowing for epitope recognition and hyporesponsive immune stimulation. Denatured type II collagen (on right) contains no tertiary and quarternary glycoprotein integrity. Epitopes of healthy undenatured type II collagen contain the correct composition and structural conformation of galactose dependent glycoprotein, as evidenced by ELISA analysis (Figure 2).

Time-dose measurements of undenatured type II collagen (UC-II) activity in simulated human gastric fluid. Five samples of UC-II were analyzed for collagen activity via ELISA analysis. Samples were digested in pepsin simulating an artificial stomach. The pepsin solution was made using 995 ml distilled water, 3.73 g KCl, 4 g HCl and 30 mg pepsin. Five collagen samples of 14.7 g each were incubated individually for 0, 15, 30, 60 and 90 min in 100 ml pepsin solution at 32ºC and pH 2.0. The digestion process was stopped by increasing the pH to 6.0 using 0.5 M NaOH solution. Both of the solid material (insoluble collagen) and the supernatant (soluble collagen) were collected and analyzed for native type II collagen using a commercially available Capture ELISA kit supplied by Chondrex LLC (Redmond, WA). Quantity of undenatured type II collagen (mg%) was determined in both supernatant soluble type II collagen and insoluble type II collagen following incubation for 0, 15, 30, 60 and 90 min at 32ºC and pH 2.0.

A pilot study to evaluate the efficacy of UC-II in human subjects. An open label pilot study was performed in five human subjects (females, ages 58-78 yr) suffering from significant joint pain, using a commercial ELISA-verified undenatured type II collagen (UC-II, InterHealth Nutraceuticals, Benicia, CA). To be eligible, patients had to meet the American College of Rheumatology criteria. Patients were excluded from the study if they had myocardial insufficiency, renal insufficiency (serum creatine >2.0 mg/dl), distrurbance of liver function alkaline phosphatase >300 units/liter, serum glutamic oxaloacetic transaminase (SGOT) >50 units/liter, or bilirubin >1.5 mg/dl), malignancy, or a considerably reduced general state of health as determined by the physician. The five subjects enrolled in this study presented a history of osteoarthritis more than rheumatoid symptomology. These subjects reported early morning stiffness, stiffness following periods of rest, pain that worsens with use of the affected joint and loss of joint range of motion and function. Weather changes from warm to cold or dry to moist were also reported as pain-enhancing factors. All patients were required to sign an informed patient consent form (PCF-1) prior to participation. The subjects were also given a questionnaire with detail protocol procedures, possible risks and benefits, etc. Two of the five subjects who suffered from osteoarthritis symptoms, were clinically diagnosed three years prior to participation in this study. The remaining three subjects reported similar symptomology. Measurements included weekly diary-format observations and qualitative feedback. Each subject received a single oral daily dose of 10 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days. Each subject was asked to rate their respective pain level on a scale of 1-10, with "10" representing "unbearable" descending to "1" noting "tolerable" prior to participation and immediately following completion of 7 days of treatment.

Results

Time-dose measurements of undenatured type II collagen (UC-II) activity in gastric fluid. Following ingestion, the UC-II glycoprotein encounters hydrochloric acid and pepsin. Dose- and time-dependent studies were conducted to determine whether these monomers are still in the triple helical form, which we confirmed by ELISA assay. Figure 3 demonstrates the time-dose measurements of UC-II activity in simulated human gastric fluid at 32°C and pH 2.0. Figure 3 clearly exhibits the UC-II activity in supernatant soluble type II collagen and insoluble type II collagen over a period of time (0-90 min). Thus, these results demonstrate that following incubation of UC-II for 90 min approximately 50% of soluble UC-II is available to the epitopes.

A pilot study to evaluate the efficacy of UC-II in human subjects. An open label pilot study was conducted in five female subjects (ages 58-78 yr) demonstrating the symptoms of significant joint pain. These subjects received a single oral daily dose of 10 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days. Each subject rated their respective pain level on a scale of 1-10 ("10" representing "unbearable" descending to "1" noting "tolerable"). The subjects rated their pain level before trial dose application and during treatment once every 7 days. Measurement of pain level in these human subjects following a 42 day supplementation of UC-II is shown in Table 1. Subject 1 perceived in her pain status throughout the open label trial. Subject 2 perceived a

reduction in pain during the sixth week of the study, while under these same conditions Subjects 3, 4 and 5 reported a reduction in their pain level during the third week of treatment. Thus, a trial dose of 10 mg UC-II was associated with a -26% reduction in perceived pain as indicated by collective of 4 of the 5 subjects [22%, 22%, 22%, 34%] (Table 1). Furthermore, there were no side effects associated with UC-II treatment. In essence, treatment with a daily oral dose of 10 mg UC-II is well tolerated and produces significant reduction in joint pain symptoms.

Discussion

Epitope Recognition. Epitopes, antigenic determinants, are structural components of an antigen molecule responsible for its specified interaction with T-cell antibody molecules elicited by the same or related antigen[23]. Epitopes of healthy undenatured type II collagen contain the correct composition and structural conformation of galactose dependent glycoprotein, as evidenced by ELISA analysis[24] (Figure 2). A novel glycosylated undenatured type II collagen material (UC-II) was developed to preserve biological activity. The presence of glycosylated "active" epitopes in the UC-II collagen matrix is confirmed by a validated ELISA test and distinguishes this form from hydrolyzed, denatured agalactosylated collagen[25]. Oral intake of 10 mg of this form of UC-II presents active epitopes, consisting of conformationally correct 3 dimensional glycosylated structures, to Peyer's Patches in the GALT[22,26]. Following ingestion, UC-II collagen glycoprotein encounters hydrochloric acid and pepsin. Dose- and Time- dependent studies show these monomers are still in their triple helical form (Figure 3) and travel down to the Peyer's Patches, to which they bind. Pepsin does not breakdown the triple helical configuration of these monomers due to biochemical limitations, so the active sites always remain intact, which is confirmed by ELISA analysis. Pepsin will not cleave bonds containing the amino acids valine, alanine or glycine[27]. The amino acid composition of native type II collagen is heavily distributed with glycine[28,29]. This glycine rich sequence ensures that pepsin will not cleave the native collagen configuration[27]. During digestion, the intact collagen fibril (a combination of collagen monomers, sugars and teloproteins) breaks down into monomeric collagen peptides (smaller glucopeptide units), exposing additional epitopes[30]. On the other hand, the teleoproteins bound to collagen molecules are susceptible to pepsin and get cleaved in the gut during digestion[31]. This allows the collagen triple helix formation to loosen slightly exposing additional active epitopes of the collagen glycoprotein, resulting in greater binding with and recognition by the Peyer's Patches[32]. These epitopes positively influence immuno-regulatory response signaling required for the development of tolerance[10,32].

Properly glycosylated epitopes did not trigger T-cell proliferation, as did modified hybrid epitopes[21]. Furthermore, Kim, et al. (2002), demonstrated that a single oral administration of poly(lactic-co-glycolic acid) (PLGA) nanoparticles induced tolerance against collagen II induced arthritis in mice[33]. Particles of PLGA were evident in the Peyer's Patches of animals for 14 days from original feeding[33]. Hyporesponsiveness results when epitopes of ingested undenatured type II collagen interact with the Peyer's Patches in the lymphoid tissues of the duodenum, triggering the complex series of immunologic events that, in the case of RA, down regulate the body's attack on it's own type II joint collagen. This research demonstrated that PLGA was well tolerated against

collagen II induced arthritis. These active epitopes meet conformational specifications of the three-dimensional glycoprotein structures required by immune surveillance to signal approval and tolerance. Antigen epitope glycosylation has been shown to play an important role in T-cell recognition and B-cell responsiveness[21,34,35]. This recognition and approval effectively turns off the up-regulated immune attack by reducing T-cell mediated inflammation, pain and swelling. UC-II has demonstrated its ability to induce tolerance, effectively reducing joint pain and swelling in RA subjects[3-6].

Conclusion

The science of glycobiology is rapidly expanding, uncapping enormous research opportunities and promising therapeutic tools[11]. It provides new insights into disease initiation, promotion and progression, especially regarding autoimmune diseases, such as RA[12]. A preponderance of the evidence suggests that all autoimmune diseases can be traced back to errors at some juncture of bioidentification, recognition and response signaling. Proper glycosylation is required for glycoconjugation, glycomolecular interconversions, biotransformations, and glycoprotein and glycolipid synthesis[11,12].

In RA, impaired galactosylation alters the requisite three-dimensional conformations of glycoproteins, including certain immune factors, like IgG and possibly even type II collagen, producing the "loss" of self-identity[12]. Alterations in glycosylation and of galactosyl structures are hallmark characteristics of RA. This loss of self identification alters recognition and response signaling during immune surveillance, inciting attack on the body's own joint collagen[13,18].

Other autoimmune disorders have been associated with faulty glycosylation as well[12,17]. This implies that certain autoimmune diseases may result when naturally occurring biomolecules are identified as foreign pathogenic antigens, due to their altered composition and structural conformation. As a result, appropriate immunological alarms are generated and aggressive defense tactics are employed against the host's own tissues[15].

Recently, safe and effective alternatives to traditional models of disease management have been used in RA[36]. Oral administration of auto-antigens has been shown to suppress a variety of experimentally induced autoimmune pathologies, including antigen-induced RA[3-6,33]. As our understanding of glyco-biochemistry increases, explanations regarding the reasons for these benefits emerge. Previous studies have shown that small doses of orally administered undenatured type II chicken collagen effectively deactivate killer T-cell attack on type II joint collagen in humans[3,22]. Our pilot study exhibited the efficacy of UC-II (10 mg/day) in effectively reducing joint pain and swelling in the human subjects without any adverse side effects. Glycosylated undenatured type II collagen contains conformationally correct "active" epitopes required to interact with Peyer's Patches in the GALT and terminate antigenic signaling of a pathogenic nature, characteristic of RA[10]. This approach provides new insights into the etiology of autoimmune inflammatory diseases and their amelioration with safe and effective treatments.

**Acknowledgement**

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FIGURE LEGENDS

Fig. 1   Electron micrograph (Magnification X 50,000) of undenatured type II collagen vs. denatured type II collagen. Undenatured type II collagen (on left) shows intact tertiary and quarternary glycoprotein integrity allowing for epitope recognition and hyporesponsive immune stimulation. Denatured type II collagen (on right) contains no tertiary and quarternary glycoprotein integrity.

Fig. 2   Undenatured type II collagen triple helix molecule exhibiting epitope positions.

Fig. 3   Time-dose measurements of UC-II activity in gastric fluid. ELISA measurements of undenatured type II collagen epitopes.
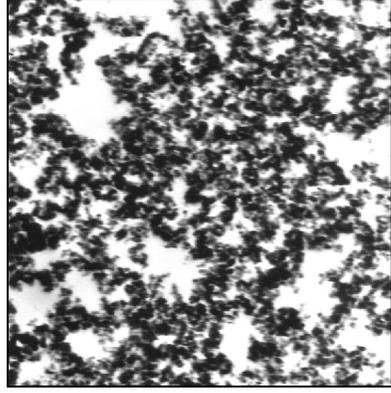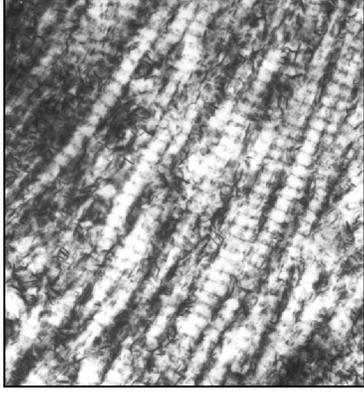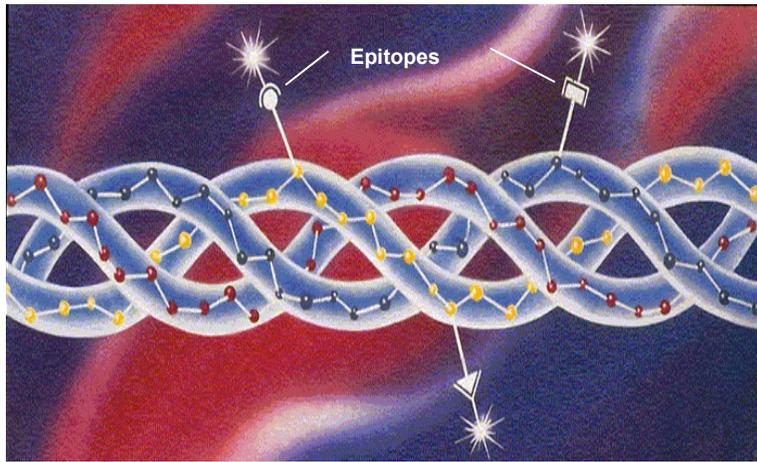
Time-dose measurements of UC-II activity in gastric fluid
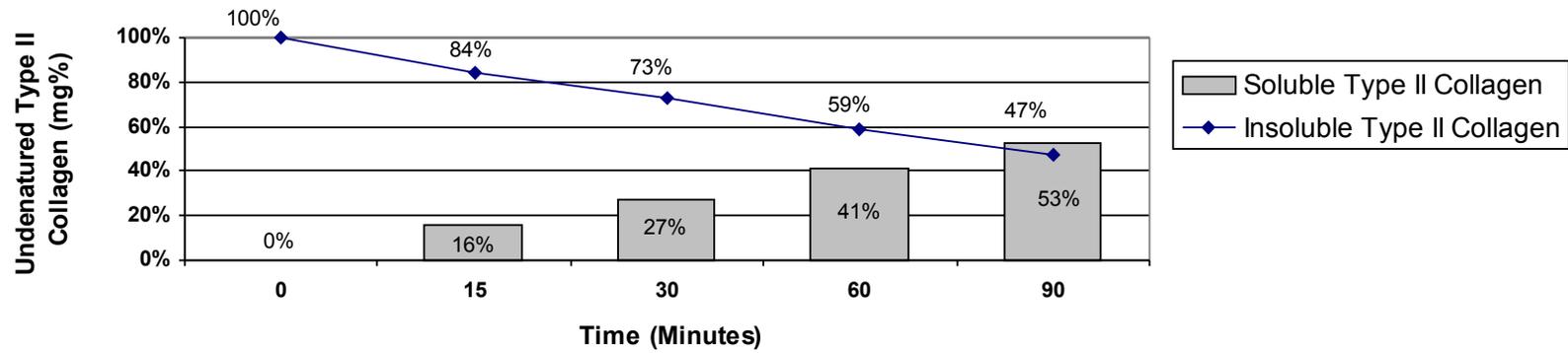**(ELISA measurements of u*ndenatured* type II collagen epitopes)**

Table 1. Measurement of pain level following a 42 day study of oral administration of Undenatured Type II Collagen (UC-II)

Table 1: Measurement of Pain Level Following a 42 day Study of Oral Administration of Undenatured Type II Collagen (UC II)

Subject #	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Reduction in Pain (%)
1	3	3	3	3	3	3	3	0
2	5	5	5	5	5	2	2	22
3	5	5	4	4	3	3	5	22
4	6	6	5	5	3	2	2	22
5	7	8	5	5	4	3	1	34

Administered Dose: A single, daily oral dose of 10 mg glycosylated Undenatured Type II Collagen (UC II) Pain Index: 10 = Unbearable, 1 = Tolerable

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Bagchi D, Misner B, Bagchi M, Kothari SC, Downs BW, Fafard RD, Preuss HG. Effects of orally administered undenatured type II collagen against arthritic inflammatory diseases: a mechanistic exploration. Int J Clin Pharmacol Res. 2002;22(3-4):101-10. PMID: 12837047 [PubMed - indexed for MEDLINE]